RNA Editing of the GP Gene of Ebola Virus is an Important Pathogenicity Factor

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Synthesis of the surface glycoprotein GP of Ebola virus (EBOV) is dependent on transcriptional RNA editing, whereas direct expression of the GP gene results in synthesis of nonstructural secreted glycoprotein sGP. In this study, we investigate the role of RNA editing in the pathogenicity of EBOV using a guinea pig model and recombinant guinea pig–adapted EBOV containing mutations at the editing site, allowing expression of surface GP without the need for RNA editing, and also preventing synthesis of sGP. We demonstrate that the elimination of the editing site leads to EBOV attenuation in vivo, explained by lower virus spread caused by the higher virus cytotoxicity and, most likely, by an increased ability of the host defense systems to recognize and eliminate virus-infected cells. We also demonstrate that expression of sGP does not affect pathogenicity of EBOV in guinea pigs. In conclusion, data obtained indicate that downregulation of the level of surface GP expression through a mechanism of GP gene RNA editing plays an important role in the high pathogenicity of EBOV.

Keywords. Ebola virus; editing site; glycoprotein GP; pathogenicity; RNA editing.

Ebola virus (EBOV) is taxonomically classified in the genus Ebolavirus, family Filoviridae, order Mononegavirales, and is an emerging zoonotic pathogen and the causative agent of lethal hemorrhagic fever in humans [1]. To date, there is no vaccine or treatment approved for human use; thus, this virus is classified as a biosafety level 4 (BSL-4) pathogen. Among ebolaviruses, Zaire Ebola virus is the most pathogenic for humans with a mortality rate reaching up to 90%. Surface glycoprotein GP is responsible for attachment and membrane fusion and is indispensable for virus replication [2, 3].

The EBOV genome is transcribed into 8 major subgenomic messenger RNAs (mRNAs) that encode 7 structural and at least 2 nonstructural proteins, and 1 truncated GP gene-specific nonstop mRNA [4, 5]. Expression of surface GP, which is encoded by 2 overlapping reading frames, requires the insertion of a nontemplate-coded adenosine residue into a number of GP-gene-specific mRNAs by a mechanism of transcriptional RNA editing [6–8]. Insertion of 2 adenosine residues at the editing site results in expression of minor nonstructural glycoprotein ssGP [6, 9, 10]. The majority of GP mRNAs are not edited and direct synthesis of other nonstructural glycoproteins of EBOV, sGP and delta peptide [6, 11–13]. Glycoproteins sGP and ssGP share their amino-terminal part with GP and are secreted from virus-infected cells. The single surface protein GP of EBOV is a type I transmembrane glycoprotein. The high level of GP glycosylation (O-linked and N-linked glycans) contributes to approximately half of the molecular mass of the protein [1, 14, 15]. Mature GP is proteolytically cleaved by the cellular protease furin into 2 subunits, GP1 and GP2, that are linked via a cysteine bond [16]. It has been demonstrated that expression of GP alone induces cell rounding and detachment, eventually followed by cell death [17, 18]. The high cytotoxicity of GP had been proposed to be the basis of the high pathogenicity of EBOV [19]. However, it has also been shown that EBOV GP is not cytotoxic when expressed constitutively at a moderate level [20]. It has been demonstrated that the cytotoxic properties of GP are associated with its overexpression and explained by its ability to cause steric hindrance and masking of cellular surface molecules, thus preventing...
Figure 1. Comparison of rEBOV/WT and rEBOV/NE in vitro and in vivo. A, Viral genome and GP gene within rEBOV/WT (WT) and rEBOV/NE (NE) are schematically presented. Guinea pig adaptation mutation (M71I, L147P, and T187I) in VP24 gene [28] are indicated by triangles. Editing site is shown by the asterisk. Wild-type editing site consists of 7 uridine residues (genome sense), and mutated editing site in rEBOV/NE contains 2 substitutions and 1 insertion (UUCCUCCUU) that prevent polymerase stuttering but allow synthesis of surface GP. B, Vero E6 cells were either mock-infected or infected with rEBOV/WT and rEBOV/NE at an MOI of 0.01, and light microscopy pictures of cells were made at several days postinfection as indicated. rEBOV/NE showed an increased cytopathic effect compared with rEBOV/WT. C, Vero E6 cells were infected with rEBOV/WT, rEBOV/NE at MOI 0.01 and samples of culture supernatant were
their normal functions [21, 22]. Significant amounts of surface GP are shed from virus-infected cells via proteolytic cleavage mediated by the cellular tumor necrosis factor (TNF–α-converting enzyme (TACE) [23]. Recently, it has been shown that shed GP triggers immune activation and increased vascular permeability [24].

A recombinant EBOV (rEBOV) containing mutations at the GP gene editing site that significantly increase the synthesis of surface GP was shown to be extremely cytotoxic due to overloading and exhausting of the cellular endoplasmic reticulum with increased amounts of the highly glycosylated protein [25]. This “no-editing” mutant of EBOV has lower growth kinetics in vitro and is less proficient at cell-to-cell spreading.

In this study, we investigated the role of RNA editing in the pathogenicity of EBOV in an animal model using guinea pig-adapted EBOV carrying “no-editing” mutations. We demonstrate that overexpression of GP results in in vivo attenuation of mutated recombinant virus. We also show that expression of sGP from an additional transcription unit does not affect pathogenicity of EBOV in guinea pigs. Data obtained indicate that downregulation of the level of surface GP expression through GP gene RNA editing (and thus a decrease in the cytotoxicity that might be caused by this protein) plays an important role in the high pathogenicity of EBOV.

**MATERIALS AND METHODS**

**Cells and Viruses**

BSR T7/5 cells (a BHK-21 cell line stably expressing T7 polymerase) were maintained in Glasgow medium (Gibco) supplemented with 10% newborn calf serum (NCS). Vero E6 and 293T cells were grown in Dulbecco’s modified Eagle medium (DMEM; Gibco) with the addition of 10% fetal calf serum (FCS). Previously described wild-type and “no-editing” variants of recombinant EBOV, based on the 1976 strain of Zaire Ebola virus, strain Mayinga, were used in in vitro and in vivo experiments [25].

**Growth Kinetics**

Subconfluent Vero E6 cells were infected with different recombinant EBOVs at a low multiplicity of infection (MOI) of 0.01. One hour postinfection (p.i.), inoculum was removed, and cells were left with DMEM media containing 2.5% FCS. Culture supernatants were collected as indicated and used for virus titration or Western blot analysis.

**Recombinant Plasmids and Generation of Recombinant EBOVs**

Details of plasmid constructions and generation of recombinant viruses are provided in the Supplementary Experimental Procedures.

**Infectious Titers**

Infectious titers were determined by median tissue culture infective dose assay (TCID<sub>50</sub>) using subconfluent Vero E6 cells as described in [26]. The titers were calculated using the Karber statistical method.

**Immunoblot Analysis**

Details of immunoblot analysis are provided in the Supplementary Experimental Procedures.

**In Vivo Studies**

Hartley guinea pigs (females, 3 weeks old) were infected intraperitonely with 500–1000 TCID<sub>50</sub> of recombinant viruses. Mock-infected controls were inoculated with DMEM. Animals were monitored for clinical manifestations and changes in body weight for 21–29 days postinfection, and were euthanized either when they reached an ethical end point in order to avoid distress and excessive suffering of animals or at the conclusion of the experiment. All animals were handled in strict accordance with good practices as defined by the French national charter on the ethics of animal experimentation. Animal work was approved by the regional ethical committee (CREEA), and experiments were performed in the Institut national de la santé et de la recherche médicale (INSERM) Jean Médecus BSL-4 laboratory in Lyon, France. Details of in vivo studies are provided in the Supplementary Experimental Procedures.

**RESULTS AND DISCUSSION**

In our earlier study [25], recombinant ebolavirus carrying mutations preventing viral polymerase stuttering at the editing site (AAGAAGAGA<sub>G</sub>, mRNA sense) and allowing surface GP expression without RNA editing have been generated and analyzed in vitro experiments. This virus demonstrated a significant increase in GP synthesis and an absence of the expression of sGP. Remarkably, this virus possessed an increased cytotoxicity in vitro and caused early death of virus-infected cells. In order to better understand the role of the GP editing site in virus replication and pathogenicity, it was thus of interest to compare...
wild-type EBOV and a no-editing mutant of EBOV in in vivo experiments using a guinea pig–adapted reverse-genetics background for the generation of recombinant viruses. Recombinant plasmids encoding full-length complementary DNA (cDNA) of EBOV coding wild-type (WT) GP and also “no-editing” (NE) mutations in the GP gene (Figure 1A) were generated essentially as described elsewhere [25, 28]. All recombinant plasmids and viruses generated in this study were carrying guinea pig adaptation mutations in the VP24 gene [28, 29], and in order to simplify the nomenclature within this paper were named with respect to GP genotype. As expected, the rEBOV/NE mutant elicits very pronounced cytopathic properties, typified by early cell death (Figure 1B), similar to that described earlier for recombinant EBOV carrying “no-editing” mutations in the GP gene and encoding wild-type VP24 [25]. A decrease in viral titers was also observed with rEBOV/NE when compared with rEBOV/WT (Figure 1C).

To address the role of RNA editing in pathogenicity, groups of guinea pigs were inoculated intraperitoneally with 1000 TCID₅₀ either with rEBOV/NE or rEBOV/WT or underwent mock infection. The animals inoculated with rEBOV/WT showed an increase in body temperature and weight loss, starting from day 3 postinfection. All animals in this group died within 6–10 days of infection (Figure 1D). Signs of disease were less pronounced in rEBOV/NE-infected animals. Some guinea pigs continued to gain weight until day 6–7 postinfection and showed a delay in the rise in body temperature (Figure 1D). On average, approximately 60%–70% of rEBOV/NE-infected animals survived infection (in 3 independent experiments). Strikingly, surviving animals started to gain weight by day 12 p.i., at which point their body temperatures had also returned to normal. Among those animals that died, some developed symptoms late postinfection, but some others died within about the same interval (7–11 days p.i.) as with rEBOV/WT-infected animals (6–10 days p.i.).

Sequence analysis of RNA isolated from the blood of infected animals showed the presence of the expected sequences at the editing site (Figure 2B) in all animals regardless of the survival pattern. The death of a small number of animals infected with rEBOV/NE at an early time point and similar to that seen with rEBOV/WT in each independent experiment suggests that host parameters must also play a role during infection and may even determine the outcome of the disease in some individuals. This is especially interesting to consider in the context of human EBOV infection in which it is often difficult to determine why some individuals survive while others appear to be incapable of controlling virus infection. Indeed, it has been suggested for humans that certain human leukocyte antigen profiles can be associated with either fatal or nonfatal filovirus infection [30].

The attenuation in viral growth observed in vitro experiments (Figure 1C) would suggest that a similar attenuation is likely to be responsible for survival of animals infected with rEBOV/NE. Indeed, amounts of viral RNA in the serum samples of rEBOV/NE-infected guinea pigs appear to be very low because nested reverse-transcription polymerase chain reaction (RT-PCR) was required to detect the presence of viral RNA (data not shown). In contrast, direct RT-PCR was sufficient for viral RNA detection in serum samples of rEBOV/WT-infected animals on day 5.

Consistent with these observations were the data of Western blot analyses of serum samples using anti-EBOV antibodies recognizing GP or VP40 proteins. It was observed that rEBOV/NE replicates to a moderate degree in guinea pigs and is released into the blood, but the level of the virus load remains remarkably low compared to that seen for rEBOV/WT-infected animals (Figure 2A, top panel). Moreover, analysis of serum samples taken from rEBOV/NE-infected animals at different times postinfection (day 3, 6, 9, and prior to euthanasia) revealed the late appearance of antigen in the blood of a surviving animal followed by an obvious reduction in antigen (Figure 2A, bottom panel, NE/6). In some way, a similar pattern was also observed for 1 animal that succumbed to infection on day 16 and which was not able to control virus replication early postinfection (Figure 2A, bottom panel, NE/3). The difference in amounts of infectious virus was also confirmed by virus titration. rEBOV/WT-infected animals revealed titers of about 2 × 10⁶ TCID₅₀/mL, while rEBOV/NE-infected animals, even those that died upon infection, at most contained 2 × 10⁵ TCID₅₀/mL. Importantly, guinea pigs that survived infection were also able to regain lost weight and to develop specific anti-EBOV antibodies (Figure 2C).

Histological analysis of liver samples showed that infection with rEBOV/WT caused multifocal necrosis, hepatocellular disruption, fatty cell degeneration, scattered hepatocellular viral inclusions, and inflammation composed of variable numbers of neutrophils and macrophages (mac387-positive cells), whereas pathological changes observed in rEBOV/NE-infected animals were much less pronounced (Figure 2D and 2E). Apparent lower replication levels and limited spread of rEBOV/NE compared to rEBOV/WT were seen in the liver and spleen of animals euthanized at day 7 p.i. (Figure 2D). On day 21, we observed almost complete virus clearance in surviving animals, as only isolated foci of viral antigen were detected in the liver (Figure 2E, NE/6).

Overall, data obtained thus far demonstrate that the loss of the editing site (and therefore loss of the virus’s ability to downregulate GP expression through a reduction in the amount of mRNA coding for surface GP) has clearly detrimental consequences for viral replication in vivo. As such, GP editing appears to be an important pathogenicity factor for EBOV.

However, it is important to consider the fact that rEBOV/NE does not express sGP. sGP is clearly the most abundant product of the GP gene and has been shown to be massively released into the blood of infected humans and animals [7]. In this context, one might suggest that the decreased pathogenicity observed with the rEBOV/NE virus could be explained by the
Figure 2. rEBOV/NE is attenuated in vivo. A, Samples of serum from guinea pigs infected as shown in Figure 1D were collected at different days post-infection, as indicated. Western blot analysis was performed using anti-GP (upper panel) and anti-GP and anti-VP40 (lower panel) antibodies. Positions of proteins and indicative numbers of animals are shown. B, Sequence analysis of viral RNA isolated from the serum samples of animals infected with either rEBOV/WT or rEBOV/NE. Sequences at the editing site are shown in circles. C, Appearance of anti-EBOV antibodies in the serum samples of survived guinea pigs infected with rEBOV/NE is shown by immunofluorescence analysis of Vero E6 cells expressing EBOV GP. D–E, Immunohistochemistry analysis of liver and spleen of guinea pigs infected as shown in Figure 1D. A number of animals were euthanized on day 7 p.i. (D). Samples of liver taken from mock-infected animals or either from rEBOV/WT- or rEBOV/NE-infected guinea pigs are shown in (E). Slides containing formalin-fixed tissues were processed and then stained with hematoxylin and eosin (HE) or stained using anti-VP40 or anti-macrophage (mac387) mouse antibody. Representative pictures of samples from each group and indicative number of animal and the day of sample collection are shown. Abbreviations: EBOV, Ebola virus; GP, glycoprotein; NE, “no-editing”; p.i., postinfection; rEBOV, recombinant EBOV; WT, wild-type.
Figure 3. Comparative analysis of rEBOV/NE/sGP (NE/sGP) and rEBOV/NE/sGP/stop (NE/sGP/stop). These viruses carry “no-editing” mutations in the GP gene and contain an additional transcription unit inserted between VP35 and VP40 genes that encode sGP. rEBOV/NE/sGP/stop possesses translational stop codon within the sGP sequence that prevents protein synthesis. Vero E6 cells were infected with recombinant viruses at an MOI of 0.01. Samples of culture medium were harvested at different time intervals as indicated, and viral release was measured either by titration assay (B) or Western blot using anti-EBOV antibody (C). Positions of viral proteins are indicated. sGP synthesis in cells either infected by EBOV/WT or rEBOV/NE/sGP was confirmed using anti-GP antibodies (C, lower panel). sGP synthesis with rEBOV/NE/sGP/stop was blocked by introduced translational stop codon. D, Guinea pigs were mock-infected or inoculated intraperitoneally with 500 TCID50 of either rEBOV/WT or rEBOV/NE/sGP or rEBOV/NE/sGP/stop. Animals were monitored for clinical manifestations (weight gain) over 29 days. E, Immunohistochemistry analysis of liver samples taken from animals infected as in (D) on the 29th day p.i.. The residual presence of EBOV antigen was detected in some animals using anti-VP40 antibody. Indicative numbers of animals are shown. Abbreviations: EBOV, Ebola virus; GP, glycoprotein; MOI, multiplicity of infection; NE, “no-editing”; p.i., postinfection; rEBOV, recombinant EBOV; sGP, secreted glycoprotein; TCID50, 50% tissue culture infective dose; WT, wild-type.
complete absence of sGP. Therefore, to address this question and in an attempt to clarify the role of sGP in EBOV pathogenicity, we generated a recombinant virus containing a full-length cDNA of rEBOV/NE harboring the sGP open reading frame (ORF) as an additional transcription unit between the genes encoding VP35 and VP40 as described in [31] (Figure 3A). In this way, the resulting recombinant virus rEBOV/NE/sGP expresses both full-length surface GP from its “no-editing” GP gene and sGP from the additional transcriptional unit. Of note, the same NE mutations that negated transcriptional editing for GP were inserted in the sGP cassette. As a control, we also generated virus in which sGP synthesis was blocked by the substitution of K135 to a stop codon (amber) in the sGP ORF (rEBOV/NE/sGP/stop). Both viruses grew in Vero E6 cells; however, the rEBOV/NE/sGP virus showed lower titers throughout the course of the experiment (Figure 3B) despite similar viral protein levels in the culture media (Figure 3C, upper panel). The presence or absence of sGP in supernatants of rEBOV/NE/sGP- or rEBOV/NE/sGP/stop-infected cells, respectively, was confirmed by Western blot analysis using anti-GP antibody (Figure 3C, lower panel).

Groups of guinea pigs were inoculated intraperitoneally with 500 infectious units (TCID50) of the rEBOV/WT or either rEBOV/NE/sGP or rEBOV/NE/sGP/stop and were monitored for disease progression by measuring the weight of animals (Figure 3D). All animals infected with rEBOV/WT died within 6 to 9 days. Animals from both rEBOV/NE/sGP and rEBOV/NE/sGP/stop groups showed temporal loss of weight but subsequently revealed gains in weight and recovered from infection. The only difference between the 2 groups of animals was a slight delay in the appearance of disease symptoms and a quicker recovery of rEBOV/NE/sGP-infected guinea pigs compared to rEBOV/NE/sGP/stop. Animals from both groups developed an antibody response, with anti-GP titers reaching 1:400.

Immunohistochemical staining of liver samples from infected animals showed the presence of viral antigen in some animals (Figure 3E, guinea pigs 2B and 3B) from the rEBOV/NE/sGP/stop group and complete clearance of antigen in the rEBOV/NE/sGP group at day 29 p.i. (Figure 3E, guinea pigs 3W and 1W). These results would suggest that sGP has a rather minor role in the high pathogenicity of EBOV, and the only effect of sGP observed in this study was a reduction in virus replication, both in cell culture and in animals. The expression of sGP by the rEBOV/NE/sGP mutant did not increase the pathogenicity of this virus, but one cannot exclude a pathogenic role for sGP in natural EBOV infection. It would also be of interest to study the potential function of sGP in the context of EBOV infection of natural hosts.

In conclusion, this study demonstrates that a “no-editing” EBOV mutant, displaying extreme cytotoxicity due to overexpression of glycoprotein GP, is less pathogenic in in vivo animal experiments. This may be explained by the early death of infected cells and therefore by limited virus production and spreading and/or the increased capacity of host defense systems to identify and eliminate virus infection in this context. Surprisingly sGP, as the major product of the GP gene, appears to have no significant influence on virus replication or pathogenicity, at least in guinea pigs. The data obtained indicate that control over the level of GP expression in infected cells is crucial for Ebola virus and intriguingly, would also seem to demonstrate that the potential for animals, and possibly also for humans, to resist this lethal infection may be higher than currently anticipated.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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